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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/763013

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP99/06131	20 August 1999	20 August 1998

TITLE OF INVENTION

METHOD FOR PRODUCING BIOSTATIN (TT-232 TRIACETATE) AND ANALOGS THEREOF

APPLICANT(S) FOR DO/EO/US

Günther BRAUM, Axel LIFFERTH and Christian BIRR

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ■ This is the **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ■ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. ■ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ■ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ■ is transmitted herewith (required only if not transmitted by the International Bureau.)
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ■ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ■ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ■ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ■ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ■ Other items or information: (a) International Search Report; (b) PCT/IB/304; PCT/IPEA/416;PCT/ISA/217;
17. ■ The follow fees are submitted: (a) Check for Filing Fee

EXPRESS MAIL NO. EL 759723731US MAILED

BASIC NATIONAL FEE (37 CFR 1.492(A)(1) - (5)):			
Search Report has been prepared by the EPO or JPO $860.00			
International preliminary examination fee paid to USPTO (37 CFR 1.482) $690.00			
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) . . . $710.00			
Neither International preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1000.00			
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00			
ENTER APPROPRIATE BASIC FEE AMOUNT =			$860.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).			$ 130.00
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	6 - 20 =	0	x $18.00 = $
Independent	3 - 3 =	0	x $80.00 = $
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $250.00 = $
	TOTAL OF ABOVE CALCULATIONS =		$990.00
Reduction of ½ for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).			$495.00
SUBTOTAL =			$495.00
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +			$
TOTAL NATIONAL FEE =			$495.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property+			$
TOTAL FEES ENCLOSED =			$495.00
		Amount to be: refunded	$
		charged	$

a. ■ A check in the amount of $495.00 (Filing Fee)) to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 50-0624 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ■ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 50-0624 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
James R. Crawford
FULBRIGHT & JAWORSKI L.L.P.
666 Fifth Avenue
New York, NY 10103
Customer No. 24972

SIGNATURE

James R. Crawford
NAME

39,155
REGISTRATION NUMBER

EXPRESS MAIL NO. EL 759723731 US Mailed February 15, 2001

HUBR 1179 (10101398)

CERTIFICATE OF EXPRESS MAIL

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Date of Deposit February 15, 2001

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231

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(Name of Depositor)

Eileen Sheffield
(Signature of Depositor)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	BRAUM, et al.
Serial Number	:	To be Assigned
Filing Date	:	February 15, 2001
For	:	METHOD FOR PRODUCING BIOSTATIN (TT-232 TRICATATE) AND ANALOGS THEREOF
Based on International Appl.	:	PCT/EP99/06131

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

February 15, 2001

PRELIMINARY AMENDMENT

Sir:

Prior to examination please amend the application as follows:

IN THE CLAIMS

Please amend the claims as follows:

Claim 1, line 5, delete "characterized in that" and substitute -- wherein -- .

Claim 2, line 2, delete "characterized in that" and substitute -- wherein -- .

Claim 3, lines 1-2, delete "or 2, characterized in that" and substitute -- wherein -- .

Claim 4, line 5, delete "characterized in that" and substitute -- wherein -- .

Claim 5, line 2, delete "characterized in that" and substitute -- wherein -- .

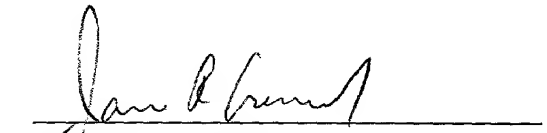
Claim 6, line 5, delete "characterized in that" and substitute -- wherein -- .

REMARKS

In this preliminary amendment, Applicants have amended the claims to eliminate multiple dependent claims. No additional fees are believed to be due. If any fees are determined to be due, authorization is hereby given to charge such fees or credit any overpayment to Deposit Account No. 50-0624.

Respectfully submitted,

FULBRIGHT & JAWORSKI, L.L.P.



James R. Crawford
Attorney for Applicants
Registration No. 39,155

FULBRIGHT & JAWORSKI, L.L.P.
666 Fifth Avenue
New York, New York 10103
(212) 318-3148
February 15, 2001

Process for preparing BIOSTATIN (TT-232 triacetate) and its analogs

Description

The present invention relates to a process for synthesizing Biostatin by means of solid phase synthesis.

The skilled person is familiar with a variety of methods for synthesizing peptides. These methods consist, on the one hand, of liquid phase methods, which originate from Shemyakin (Tetrahedron Lett. (1965), 2323 f.) and, on the other hand, of solid phase methods, which were described for the first time by Merrifield (R.B. Merrifield, J. Am. Chem. Soc. 85 (1963) 2149).

Since then, the methods of solid phase synthesis and liquid phase synthesis have been subject to further development and have been considerably improved; in this regard, the reader is referred, for example, to "Peptide, Chemie und Biologie [Peptides, chemistry and biology]", Hans Dieter Jakubke, Spektrum Akademischer Verlag, Heidelberg-Berlin-Oxford, 1996, ISBN 3-8274-0000-7. This textbook describes methods of both classical and Merrifield peptide synthesis. At present, peptide synthesis in solution is primarily employed for synthesizing the peptides. However, particularly when synthesizing peptides which are to form at least one disulfide bridge, the synthesis in solution suffers from the disadvantage, which is inherent in the method, that this disulfide bridge has to be formed by oxidation at high dilution. In the classical method of synthesizing peptides in solution, this is necessary in order to bring about the requisite spatial separation of the individual reaction centers and thereby permit effective cyclization.

The peptide Biostatin (TT-232) is an analog of somatostatin and exhibits a powerful antitumor activity both in vitro and in vivo.

Somatostatin is a naturally occurring tetradecapeptide which inhibits the formation of growth hormone and the secretion of other endocrine molecules such as glucagon, insulin and gastrin. Somatostatin inhibits or regulates some cell functions, and it has furthermore been observed that it displays significant endogenous antiproliferative activity. In addition, somatostatin and its analogs have been shown to have an inhibitory effect on tumors. The last few years have seen the development of a number of somatostatin analogs which act for longer periods than does the native hormone and which possess superior antitumor activity. A great deal of effort has therefore been expended in developing tumor-selective somatostatin analogs, with the ability to prepare the analogs easily also being a particular consideration.

One of these analogs is a molecule which possesses a 5-membered ring structure and which has the following sequence:
D-Phe-Cys-Tyr-D-Trp-Lys-Cys-Thr-NH_2.

The molecule was named TT-232 or Biostatin. While this somatostatin analog has practically no inhibitory effect on the release of growth hormone, it exhibits powerful antitumor activity both in vivo and in vitro and induces apoptosis. The compound inhibits the tyrosine kinase activity of a variety of human intestinal tumor cell lines, with this inhibition being in very good agreement with the observed inhibition of the cell proliferation.

The preparation of octapeptide and heptapeptide derivatives is described, for example, in EP-A-0 505 680. However, in order to achieve efficient cyclization by way of the two cysteine residues in this publication, the peptide is first of all separated off from the solid phase, after which the solution is diluted extensively and the oxidation is then effected. This type of preparation therefore requires further steps of concentration and purification.

The object of the present invention was therefore to make available a process by which Biostatin can be obtained particularly readily and with a particularly high yield of released peptideamide following the disulfide oxidation.

Another object was to enable Biostatin to be prepared in such a way that the resulting product can be worked up easily.

These objects regarding the synthesis of Biostatin (TT 232) are achieved, in a first process according to the invention, by means of solid phase synthesis on a polymeric support, by the peptide being synthesized stepwise using protective group-derivatized amino acids, after which the protective groups are eliminated and the peptide is released from the solid phase, with the disulfide bridge being closed by oxidation of the completely or partially synthesized peptide in the presence of a suitable solvent, as long as the peptide is still bound to the solid phase.

The solid phase synthesis which is used within the context of the process according to the invention can be carried out in a manner known to the skilled person. The solid phase materials which are suitable for this purpose, and the reagents and buffers which are required, and the reaction conditions and the

protective groups which are to be employed for the amino acids, are known to the skilled person.

The process according to the invention is based on the observation that the spatial separation of the reaction centers, when forming the disulfide bridges in Biostatin, is adequately ensured if the oxidation takes place while the peptide is still bound to the solid phase.

Within the context of the invention, it is possible both to effect oxidation, and thereby formation of the disulfide bridge, directly following synthesis of that part of the Biostatin which contains the sulfhydryl groups which are to be bridged, and then to complete the synthesis of the peptide, and also to synthesize the complete peptide first of all and carry out the oxidation after that. However, the crucial point is that the oxidation has to take place while the peptide is bound to the solid phase.

Within the context of the present invention, preference is given to the oxidation being carried out before the peptide protective groups are eliminated.

All the oxidizing agents which have also already been known previously for methods carried out in solution may be employed for the oxidation. The skilled person is therefore familiar with suitable oxidizing agents. Examples of such oxidizing agents are salts of silver, mercury or thallium, and iodine, peroxides or oxygen. These oxidizing agents are employed in the presence of a suitable solvent or solvent mixture.

Within the context of the present invention, particular preference is given to using iodine, for example in solution in acetic acid or in a solvent based on N,N-dimethylformamide, as the oxidizing agent.

After the oxidation has been concluded, the polymer-bound peptide is washed a number of times with various solvents or solvent mixtures. N,N-Dimethylformamide, methanol, acetic acid and water, or else also solutions of complexing reagents or reducing agents, such as, in particular, thiosulfate or ascorbic acid, can, for example, be employed for this purpose.

The process according to the invention is advantageously carried out on a solid phase which possesses an acid-labile anchoring group (acid labile anchoring bond, ALAB). A polymer, in particular polystyrene, is particularly preferably employed as the solid phase. It is also advantageously possible to use modified resins such as aminomethylpolystyrene (AMPS), benzhydrylaminepolystyrene (BHAPS) and methylbenzhydrolaminopolystyrene (MBHAPS). In this connection, the solid phase can be employed in a form which is customary for solid phase synthesis. Preference is given to employing the solid phase in the form of spheres, i.e. so-called beads.

Suitable anchoring groups are anchors which are customary in solid phase chemistry and which allow the peptide to be cleaved off the polymeric support in a simple manner. In the context of the invention, particular preference is given to anchoring groups which enable the peptide to be cleaved off as an amide. Examples of polymers which are derivatized with an acid-labile anchoring group (ALAB-P) are 5-(9-amino)xanthen-2-yl)oxyveryl-4'-methylbenzhydrylamino-polystyrene and 4-(2',4'-dimethoxyphenyl)aminomethyl-phenoxyacetyl-4"-methylbenzhydrylaminopolystyrene.

Particularly preferred anchoring groups are, additionally, 4-hydroxymethylbenzoic acid (HBMA), 9-aminoxanthenyl-3-hydrol (Xant) or p[(R,5)-α-(1-(9H-

fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid [MEOBP]. Within the context of the invention, greatest preference is given to the Xant and MEOBP groups.

In the process according to the invention, the synthesis is preferably carried out using the Fmoc/tert-butyl strategy. This means that the amino acids which are required for synthesizing the peptide are derivatized on the amino group with an Fmoc protective group and on the side chain groups with tert-butyl groups. In this connection, the Fmoc protective group is a temporary protective group since it is eliminated during the formation of the peptide and only one Fmoc group remains at the N terminus of the synthesized and solid phase-bound peptide.

The sulfhydryl groups of the cysteines are advantageously derivatized with trityl or Acm protective groups. In addition, particular preference is given to that amino acid which is the last N-terminally in the sequence synthesis being employed as an alpha-N Boc-protected amino acid derivative.

The synthesis process according to the invention involves performing the following steps:

1. Loading the polymeric support with the anchor and/or the first amino acid derivative
2. Synthesizing the peptide sequence
3. Linking the disulfide bridge
4. Cleaving the peptide off the polymeric support and/or eliminating the protective groups
5. Eliminating the protective groups (provided this has not already taken place under 4.).

Methods which are known from the literature, for example the addition of dilute piperidine solution, can

be employed for eliminating the protective groups which are contained in the synthesized peptide.

The peptides are likewise cleaved off the polymeric support using methods which are known per se. In the case of the acid-labile anchoring groups, the cleavage takes place under acid conditions, particularly preferably using concentrated or dilute trifluoroacetic acid.

As a rule, the protective groups attached to the peptides which have been released from the solid phase are likewise eliminated by adding acid, preferably once again using trifluoroacetic acid. After the peptides have been cleaved off, additional purification and/or concentration steps can be carried out if desired. In this connection, purification can advantageously be effected by means of preparative HPLC.

In a particularly preferred embodiment, the synthesis in accordance with the process according to the invention advantageously starts from Fmoc-threonine (tert-butyl ether) amide, which is covalently bonded to a polystyrene solid phase by way of an acid-labile xanthenyl anchoring group.

Subsequently, the individual protected amino acids are added with the formation of a solid phase-bound, protected peptide. In order to form the disulfide bridge, the heptapeptide is then oxidized on the solid phase by adding iodine/N,N-dimethylformamide or acetic acid and the cyclized heptapeptide is released from the support by treatment with acid. All the protective groups on the peptide side chains are eliminated at the same time.

The present invention furthermore relates to a process for synthesizing Biostatin (TT-232) by means of peptide

synthesis in solution, by synthesizing the peptide stepwise using protective group-derivatized amino acids, with the disulfide bridge being closed by oxidizing the completely or partially synthesized peptide in the presence of a suitable solvent and the Biostatin being obtained after the solvent has been removed and the product has, where appropriate, been washed.

The process according to the invention makes it possible to use peptide synthesis in solution to prepare Biostatin very efficiently and simply when compared with the state of the art. In particular it is possible to obtain high yields of the product, in a simple manner, when the process is conducted in the preferred manner using amino acids, for the peptide synthesis, which are derivatized with Ddz(3,5-dimethoxybenzyl-α,α-dimethyloxycarbonyl or 2[(3,5-dimethoxyphenyl)-2-oxycarbonyl]propyl) as the protective group. In addition, the process according to the invention has the advantage that the oxidation can readily be effected after the peptide synthesis has been completed. While preference is given, within the context of the present invention, to carrying out the oxidation before all protective groups have been eliminated, it is also possible for the process to be conducted such that the protective groups are eliminated prior to the oxidation, even if the yields are somewhat lower with this variant.

A particular advantage of the process according to the invention is that, after the oxidation, and thus intramolecular ring closure by way of the two cysteine residues, has taken place, the reaction solution can be evaporated off, with the product being obtained in this way. Where appropriate, the product is also washed, for example with ether, and after that filtered off with suction once again and dried.

In the process according to the invention, preference is given to carrying out the following synthesis steps consecutively:

1. Coupling Ddz-protected Cys(Acm) to tert-butyl-protected threonine
2. Replacement of the Ddz protective group with trifluoroacetic acid,
3. Addition of a Ddz-protected lysine (Z),
4. Replacement of the Ddz protective group with trifluoroacetic acid,
5. Addition of the Ddz-protected D-Trp
6. Removal of the Ddz protective group with trifluoroacetic acid
7. Addition of the Ddz-protected Tyr (Tbu)
8. Removal of the Ddz protective group and replacement with trifluoroacetic acid
9. Addition of the Ddz-protected Cys (Acm)
10. Remcval of the Ddz protective group with trifluoroacetic acid
11. Addition of the Boc-protected D-Phe
12. Replacement of the Boc with trifluoroacetic acid
13. Oxidation and working up of the product by evaporating off the solvent and washing.

Whereas the oxidation is carried out with the completely protected peptide in preferred embodiments, the protective groups are already partially removed in step 11 in the other embodiment, such that it is only the Acm groups on the cysteine which still remain.

The process according to the invention which is a second part of the subject matter of the present invention makes it possible to synthesize the peptide in solution in a simple manner, with both the oxidation and the working-up being very easy to carry out. A particularly high yield, of from approx. 70 to 80% of

theory, is obtained by oxidizing the Biostatin while it is still tert-butyl-protected.

Other embodiments of the process according to the invention are described in Examples 4 and 5.

The following examples are intended to further clarify the invention.

Example 1:

Step 1, elimination of the Fmoc protective group from the linker

395 g of Fmoc-MEOBP-MBHA resin (loading, 0.84 mmol/g) are transferred, using 1.5 l of N,N-dimethylformamide, into a reaction vessel which is equipped with a bottom frit and mixed by tumbling; after 5 minutes, the liquid is filtered off with suction. The tumbling movement is maintained during all the washing and reaction steps. There then follows a DMF washing step; for this, 1.5 l of N,N-dimethylformamide are added to the reaction vessel and the N,N-dimethylformamide is filtered off with suction after 5 minutes. 1.5 l of 50% piperidine in N,N-dimethylformamide (v/v) are then added. After 5 minutes, the piperidine solution is filtered off with suction and a further 1.5 l of 50% piperidine in N,N-dimethylformamide (v/v) are added. After 30 minutes, the piperidine solution is filtered off with suction and a further 1.5 l of 50% piperidine in N,N-dimethylformamide (v/v) are added. After 5 minutes, the piperidine solution is filtered off with suction and 5 DMF washing steps (see above) are then carried out.

Step 2, coupling of Fmoc-Thr(tBu)

The following solutions are prepared during the last DMF washing steps of the Fmoc elimination (step 1): 267.1 g (672 mmol) of Fmoc-Thr(tBu) in 375 ml of N,N-dimethylformamide, 104.4 g (672 mmol) of HOBt*H_2O in 250 ml of N,N-dimethylformamide and 215.8 g of TBTU in 750 ml of N,N-dimethylformamide. When they have been prepared, the solutions are added to the deblocked linker polymer (Step 1) in the reaction vessel; after that, 228.7 ml of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and then examined for the presence of free amino groups. The Kaiser test shows complete conversion; 4 DMF washing steps (see above) are carried out.

Step 3, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 1, with the introductory swelling procedure being dispensed with in view of the fact that the resin is already moistened with DMF.

Step 4, coupling of Fmoc-Cys(Trt)

The following solutions are prepared during the last DMF washing steps of the Fmoc elimination (Step 3): 393.6 g (672 mmol) of Fmoc-Cys(Trt) in 375 [lacuna] of N,N-dimethylformamide, 104.4 g (672 mmol) of HOBt*H_2O in 250 ml of N,N-dimethylformamide and 215.8 g of TBTU in 750 ml of N,N-dimethylformamide. When they have been prepared, the solutions are added to the deblocked linker polymer (Step 3) in the reaction vessel; after that, 228.7 ml of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows incomplete conversion; a secondary coupling is carried out using the following solutions:

196.8 g (336 mmol) of Fmoc-Cys(Trt in 250 ml of N,N-dimethylformamide, 52.2 g (336 mmol) of HOBt*H_2O in 125 ml of N,N-dimethylformamide and 107.9 g of TBTU in 375 ml of N,N-dimethylformamide. These solutions are added to the reaction vessel, after which 114.3 ml of DIEA are added. After a reaction period of 1 hour, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows complete conversion; 4 DMF washing steps (see above) are carried out.

Step 5, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 6, coupling of Fmoc-Lys(Boc)

The following solutions are prepared during the last DMF washing steps of the Fmoc elimination (Step 5): 314.9 g (672 mmol) of Fmoc-Lys(Boc) in 500 ml of N,N-dimethylformamide, 104.4 g (672 mmol) of HOBt*H_2O in 250 ml of N,N-dimethylformamide and 215.8 g of TBTU in 750 ml of N,N-dimethylformamide. When they have been prepared, the solutions are added to the deblocked linker polymer (Step 5) in the reaction vessel; after that, 228.7 ml of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows incomplete conversion; a secondary coupling is carried out using the following solutions: 157.4 g (336 mmol) of Fmoc-Lys(Boc) in 250 ml of N,N-dimethylformamide, 52.2 g (336 mmol) of HOBt*H_2O in 125 ml of N,N-dimethylformamide and 107.9 g of TBTU in 375 ml of N,N-dimethylformamide. These solutions are added to the reaction vessel, after which 114.3 ml of DIEA are added. After a reaction period of 1 hours, a resin sample is removed and washed and examined for the

presence of free amino groups. The Kaiser test shows complete conversion; 4 DMF washing steps (see above) are carried out.

Step 7, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 8, coupling of Fmoc-D-Trp

The following solutions are prepared during the last DMF washing steps of the Fmoc elimination (Step 7): 286.8 g (672 mmol) of Fmoc-D-Trp in 500 ml of N,N-dimethylformamide, 104.4 g (672 mmol) of HOBt*H_2O in 250 ml of N,N-dimethylformamide and 215.8 g of TBTU in 750 ml of N,N-dimethylformamide. When they have been prepared, the solutions are added to the deblocked linker polymer (Step 7) in the reaction vessel; after that, 228.7 ml of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows incomplete conversion; a secondary coupling is carried out using the following solutions: 143.3 g (336 mmol) of Fmoc-D-Trp in 250 ml of N,N-dimethylformamide, 52.2 g (336 mmol) of HOBt*H_2O in 125 ml of N,N-dimethylformamide and 107.9 g of TBTU in 375 ml of N,N-dimethylformamide. These solutions are added to the reaction vessel, after which 114.3 ml of DIEA are added. After a reaction period of 1 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows complete conversion; 4 DMF washing steps (see above) are carried out.

Step 9, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 10, coupling of Fmoc-Try(tBu)

The following solutions are prepared during the last DMF washing steps of the Fmoc elimination (Step 9): 308.8 g (672 mmol) of Fmoc-Tyr(tBu) in 500 ml of N,N-dimethylformamide, 104.4 g (672 mmol) of HOBt*H_2O in 250 ml of N,N-dimethylformamide and 215.8 g of TBTU in 750 ml of N,N-dimethylformamide. When they have been prepared, the solutions are added to the deblocked linker polymer (Step 9) in the reaction vessel; after that, 228.7 ml of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows incomplete conversion; a secondary coupling is carried out using the following solutions: 154.4 g (336 mmol) of Fmoc-Tyr(tBu) in 250 ml of N,N-dimethylformamide, 52.2 g (336 mmol) of HOBt*H_2O in 125 ml of N,N-dimethylformamide and 107.9 g of TBTU in 375 ml of N,N-dimethylformamide. These solutions are added to the reaction vessel, after which 114.3 ml of DIEA are added. After a reaction period of 1 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows complete conversion; 4 DMF washing steps (see above) are carried out.

Step 11, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 12, coupling of Fmoc-Cys(Trt)

The following solutions are prepared during the last DMF washing steps of the Fmoc elimination (Step 1): 393.6 g (672 mmol) of Fmoc-Cys(Trt) in 500 ml of N,N-dimethylformamide, 104.4 g (672 mmol) of HOBt*H_2O in 250 ml of N,N-dimethylformamide and 215.8 g of TBTU in 750 ml of N,N-dimethylformamide. When they have been prepared, the solutions are added to the deblocked linker polymer (Step 11) in the reaction vessel; after that, 228.7 ml of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows incomplete conversion; a secondary coupling is carried out using the following solutions: 196.8 g (336 mmol) of Fmoc-Cys(Trt) in 250 ml of N,N-dimethylformamide, 52.2 g (336 mmol) of HOBt*H_2O in 125 ml of N,N-dimethylformamide and 107.9 g of TBTU in 375 ml of N,N-dimethylformamide. These solutions are added to the reaction vessel, after which 114.3 ml of DIEA are added. After a reaction period of 1 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows complete conversion; 4 DMF washing steps (see above) are carried out.

Step 13, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 14, coupling of Boc-D-Phe

The following solutions are prepared during the last DMF washing steps of the Fmoc elimination (Step 1): 178.3 g (672 mmol) of Boc-D-Phe in 500 ml of N,N-dimethylformamide, 104.4 g (672 mmol) of HOBt*H_2O in 250 ml of N,N-dimethylformamide and 215.8 g of TBTU

in 750 ml of N,N-dimethylformamide. When they have been prepared, the solutions are added to the deblocked linker polymer (Step 11) in the reaction vessel; after that, 228.7 ml of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows incomplete conversion; a secondary coupling is carried out using the following solutions: 89.1 g (336 mmol) of Boc-D-Phe in 250 ml of N,N-dimethylformamide, 52.2 g (336 mmol) of HOBt*H_2O in 125 ml of N,N-dimethylformamide and 107.9 g of TBTU in 400 ml of N,N-dimethylformamide. These solutions are added to the reaction vessel and 114.3 ml of DIEA are then added. After a reaction period of 1 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows complete conversion; 4 DMF washing steps (see above) are carried out.

Step 15, reaction with Boc_2O

4 l of N,N-dimethylformamide are added to the product resulting from Step 14 and the mixture is agitated for 20 minutes. 400 g of Boc_2O are then added; after 5 minutes, 3 portions of DIEA, each of 200 ml volume, are then added at 5 minute intervals. After 1000 minutes, the mixture is filtered with suction and there then follow 5 DMF washing steps (see above), of 3 l of DMF in each case, and 3 analogous MeOH washing steps, with 2.5 l of MeOH being employed in each case. After drying for 16 hours under high vacuum, 833 g of polymer-bound peptide are obtained.

Step 16, linking the disulfide bridge

A solution comprising 416.5 g of iodine in 6 l of N,N-dimethylformamide is added to 833 g of polymer-bound peptide (0.37 mmol of peptide/g of peptide-

support conjugate) obtained from Step 15. After 60 minutes, the mixture is filtered with suction and 8 l of N,N-dimethylformamide are then added. The mixture is filtered with suction after 60 minutes and there then follow 4 DMF washing steps (see above) of 8 l volume in each case. The following procedure is then carried out 3 times: 8 l of N,N-dimethylformamide and 2 l of 10% $Na_2S_2O_3$ solution are added. After 5 minutes, the mixture is filtered with suction and there then follow 3 washing steps with a mixture comprising 8 l of N,N-dimethylformamide and 2 l of water, and then 2 DMF washing steps of 8 l volume in each case. The resin is then washed in each case 3 times with water, methanol, water and methanol and finally dried overnight under high vacuum. Yield 672.4 g (0.45 mmol of peptide/g of peptide-support conjugate).

Step 17, cleaving off the polymer

A solution of in each case 120 ml of m-cresol and water in 6 l of trifluoroacetic acid (cleavage reagent) is added to 672.4 g of polymer-bound peptide from Step 16 and the mixture is shaken at room temperature for 30 minutes. After that, it is filtered with suction and fresh cleavage reagent is added to the resin. The first secondary cleavage is filtered off with suction after 30 minutes, and secondary cleavages of one or two hours duration then follow. The respective filtrates are evaporated in a rotary evaporator at 30°C water bath temperature using a water vacuum pump. The residue is stirred up in 3 l of ether, filtered off with suction on a P3 frit and washed 3 times with 1.5 l of ether on each occasion. After drying for 16 hours under high vacuum, a total of 231.85 g of peptide is obtained.

Yield: 13.4% of theory over all the steps; 14.8% based on the elimination.

Example 2:

Step 1, elimination of the Fmoc protective group from the linker

3.64 g of Fmoc-XANT resin (loading, 0.55 mmol/g) are transferred, using 25 ml of N,N-dimethylformamide, into a reaction vessel which is equipped with a bottom frit and the mixture is shaken; after 5 minutes, it is filtered with suction. The shaking is maintained during all the washing and reaction steps. There then follows a DMF washing step; for this, 25 ml of N,N-dimethylformamide are added to the reaction vessel; after 5 minutes, the N,N-dimethylformamide is filtered off with suction. 25 ml of 50% piperidine in N,N-dimethylformamide (v/v) are added. After 5 minutes, the piperidine solution is filtered off with suction and 25 ml of 50% piperidine in N,N-dimethylformamide (v/v) are added once again. After 25 minutes, the piperidine solution is filtered off with suction and 25 ml of 50% piperidine in N,N-dimethylformamide (v/v) are added. After 5 minutes, the piperidine solution is filtered off with suction and 5 DMF washing steps (see above) are then carried out.

Step 2, coupling of Fmoc-Thr(tBu)

During the last DMF washing steps of Fmoc elimination (Step 1), 2.39 g (6 mmol) of Fmoc-Thr(tBu), 1.12 g (7.2 mmol) of HOBt*H_2O and 2.12 g (6.6 mmol) of TBTU are dissolved in 15 ml of N,N-dimethylformamide. This solution is added to the deblocked linker polymer (Step 1) in the reaction vessel, after which 2.04 ml (12 mmol) of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows complete conversion; 5 DMF washing steps (see above) are carried out.

Step 3, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described under Step 1, with the introductory swelling procedure being dispensed with in view of the fact that the resin is moistened with DMF.

Step 4, coupling of Fmoc-Cys(Trt)

During the last DMF washing steps of the Fmoc elimination (Step 3), 3.51 g (6 mmol) of Fmoc-Cys(Trt), 1.12 g (7.2 mmol) of HOBt*H_2O and 2.12 g (6.6 mmol) of TBTU are dissolved in 15 ml of N,N-dimethylformamide. This solution is added to the deblocked linker polymer (Step 3) in the reaction vessel, after which 2.04 ml (12 mmol) of DEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows complete conversion; 5 DMF washing steps (see above) are carried out.

Step 5, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 6, coupling of Fmoc-Lys(Boc)

During the last DMF washing steps of the Fmoc elimination (Step 5), 2.81 g (6 mmol) of Fmoc-Lys(Boc), 1.12 g (7.2 mmol) of HOBt*H_2O and 2.12 g (6.6 mmol) of TBTU are dissolved in 15 ml of N,N-dimethylformamide. This solution is added to the deblocked linker polymer (Step 5) in the reaction vessel, after which 2.04 ml (12 mmol) of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The

Kaiser test shows complete conversion; 5 DMF washing steps (see above) are carried out.

Step 7, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 8, Coupling of Fmoc-D-Trp

During the last DMF washing steps of Fmoc elimination (Step 7), 2.56 g (6 mmol) of Fmoc-D-Trp, 1.12 g (7.2 mmol) of HOBt*H_2O and 2.12 g (6.6 mmol) of TBTU are dissolved in 15 ml of N,N-dimethylformamide. This solution is added to the deblocked linker polymer (Step 7) in the reaction vessel, after which 2.04 ml (12 mmol) of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows complete conversion; 5 DMF washing steps (see above) are carried out.

Step 9, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 10, coupling of Fmoc-Tyr(tBu)

During the last DMF washing steps of the Fmoc elimination (Step 9), 2.76 g (6 mmol) of Fmoc-Tyr(tBu), 1.12 g (7.2 mmol) of HOBt*H_2O and 2.12 g (6.6 mmol) of TBTU are dissolved in 15 ml of N,N-dimethylformamide. This solution is added to the deblocked linker polymer (Step 9) in the reaction vessel, after which 2.04 ml (12 mmol) of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The

Kaiser test shows complete conversion; 5 DMF washing steps (see above) are carried out.

Step 11, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 12, coupling of Fmoc-Cys(Trt)

During the last DMF washing steps of Fmoc elimination (Step 11), 3.51 g (6 mmol) of Fmoc-Cys(Trt), 1.12 g (7.2 mmol) of HOBt*H_2O and 2.12 g (6.6 mmol) of TBTU are dissolved in 15 ml of N,N-dimethylformamide. This solution is added to the deblocked linker polymer (Step 11) in the reaction vessel, after which 2.04 ml (12 mmol) of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The Kaiser test shows complete conversion; 5 DMF washing steps (see above) are carried out.

Step 13, elimination of the Fmoc protective group

The Fmoc protective group is eliminated as described in Step 3.

Step 14, coupling of Boc-D-Phe

During the last DMF washing steps of Fmoc elimination (Step 13), 1.59 g (6 mmol) of Boc-D-Phe, 1.12 g (7.2 mmol) of HOBt*H_2O and 2.12 g (6.6 mmol) of TBTU are dissolved in 15 ml of N,N-dimethylformamide. This solution is added to the deblocked linker polymer (Step 13) in the reaction vessel, after which 2.04 ml (12 mmol) of DIEA are added. After a reaction period of 2 hours, a resin sample is removed and washed and examined for the presence of free amino groups. The

Kaiser test shows complete conversion; 5 DMF washing steps (see above) are carried out.

Step 15, linking the disulfide bridge

A solution of 2.5 g of iodine in 50 ml of N,N-dimethylformamide is added to 5 g of polymer-bound peptide (0.30 mmol of peptide/g of peptide-support conjugate) obtained from Step 14. After 60 minutes, the mixture is filtered with suction and 50 ml of N,N-dimethylformamide are added. After 60 minutes, the mixture is filtered with suction and there then follow 4 DMF washing steps (see above) with 50 ml in each case. The following procedure is carried out 3 times: 16 ml of N,N-dimethylformamide and 4 ml of a 10% $Na_2S_2O_3$ solution are added. After 5 minutes, the mixture is filtered with suction and there then follow 3 DMF washing steps. The resin is then washed, in each case 3 times, with water, methanol, water and methanol and dried overnight under high vacuum. Yield 4.1 g (0.34 mmol of peptide/g of peptide-support conjugate).

Step 16, cleaving off the polymer and then eliminating the protective groups

1 g of polymer-bound peptide is treated 10 times for in each case 10 minutes with in each case 5 ml of 1% trifluoroacetic acid in dichloromethane. The peptide is then cleaved off by being treated 3 times with 5% trifluoroacetic acid in dichloromethane. The cleavage solutions are pooled and evaporated and the residue is stirred for 30 minutes in 2.5 ml of trifluoroacetic acid. The peptide is then precipitated in 20 ml of ether and filtered off with suction on a P3 frit; it is then washed 3 times with 10 ml of ether on each occasion. After drying for 16 hours under high vacuum, 332 mg of peptide are obtained.

Yield: 23.9% of theory over all the steps, 33.0% based on the elimination

Example 3: Disulfide oxidation using thallium trifluoroacetate

A solid phase synthesis is first of all carried out as described in Example 1 but on a BHAPS (loading, 1 mmol/g) which is loaded with MEOBP linker. FmocCys(Acm) is used in place of Fmoc-Cys(Trt).

136 mg of $Tl(TFA)_3$ are dissolved in 1 ml of N,N-dimethylformamide (oxidizing solution); 0.5 g of polymer-bound peptide (0.38 mmol of peptide/g of peptide-support conjugate) are shaken together with 3 ml of N,N-dimethylformamide for 5 minutes, after which 0.725 ml of oxidizing solution is added. After the mixture has been shaken at 25°C for 30 minutes, it is filtered with suction and washing takes place with in each case 5 ml of the following solvents: 3× N,N-dimethylformamide, 3× MeOH, 1 × 10% HAc in N,N-dimethylformamide, 1 × 5% EDTA in H_2O, 1 × 10% HAc in N,N-dimethylformamide, 1 × 5% EDTA in H_2O, 1 × 10% HAc in N,N-dimethylformamide, 1 × 5% EDTA in H_2O, 1 × H_2O, 3 x 10% HAc in MeOH, 3 × 10% HAc in H_2O, 3 × N,N-dimethylformamide, 3 × MeOH, 3 × H_2O, 3 × MeOH. The resin is dried overnight under high vacuum. Yield 420 mg (0.40 mmol of peptide/g of peptide-support conjugate).

10 ml of trifluoroacetic acid are added to 0.25 ml of triethylsilane (thereby forming the cleavage reagent). 0.4 g of polymer-bound peptide is shaken with 3 ml of cleavage reagent for 30 minutes and the mixture is then filtered with suction; 2.5 ml of cleavage reagent are then added. After 30 minutes of shaking, the mixture is filtered with suction and the resin is treated for a further 1 and 2 hours, respectively, with in each case

2.5 ml of cleavage reagent. The filtrates are evaporated and the residues are in each case triturated with 3 ml of ether; the precipitates which result in this connection are filtered off with suction on a P3 frit and washed 3 times with 2 ml of ether on each occasion. After drying for 16 hours under high vacuum, a total of 119 mg of peptide is obtained.

Yield: 8.6% of theory over all the steps,
8.6% based on the elimination

Example 4: Synthesis of TT232 in solution

1. Ddz-Cys(Acm)-Thr(tBu)-NH_2

22.8 g (55 mmol) of Ddz-Cys(Acm) and 9.32 g (60 mmol) of HOBT are dissolved in 300 ml of ethyl acetate in a 1 l round-bottomed flask. 18.46 g (57.5 mmol) of TBTU and 27.45 ml (0.25 mol) of NMM are added to the stirred solution. After the mixture has been stirred for a further 5 minutes, 8.71 g (50 mmol) of Thr(tBu)-NH_2 are added. The mixture is stirred overnight. 150 ml of benzine are added to the reaction solution and this mixture is then washed with 1 × 100 ml of deionized water, 3 × 100 ml of saturated $NaHCO_3$ solution, 1 × 100 ml of deionized water, 3 × 100 ml of 0.1 N HCl, 1 × 100 ml of brine and 1 × 100 ml of saturated $NaHCO_3$ solution; it is then dried over 10 g of magnesium sulfate and evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature. 25.2 g of amorphous product are obtained (yield: 88% of theory).

2. TFA*Cys(Acm)-Thr(tBu)-NH_2

18.3 g (32 mmol) of Ddz-Cys(Acm)-Thr(tBu)-NH_2 are dissolved in 200 ml of 5% trifluoroacetic acid in DCM and the solution is stirred at RT for 1 h. After 200 ml

of toluene have been added, the mixture is evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature and then codistilled with 100 ml of ethyl acetate. After drying under high vacuum, the residue is taken up in a mixture composed of 30 ml of deionized water, 15 ml of ethyl acetate and 30 ml of diethyl ether. The phases are separated in a separating funnel and the organic phase is extracted a further 2× with 10 ml of deionized water on each occasion. The combined aqueous phases are washed with 3 × 10 ml of ethyl acetate/diethyl ether 1:2(v/v) and lyophilized. 11.8 g of amorphous product are obtained (yield: 80% of theory).

3. Ddz-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2

12 g (26 mmol) of TFA*Cys(Acm)-Thr(tBu)-NH_2, 14.34 g (28.5 mmol) of Ddz-Lys(Z) and 4.84 g (31.1 mmol) of HOBT are dissolved in 100 ml of ethyl acetate in a round-bottomed flask. 9.58 g (29.8 mmol) of TBTU and 14.3 ml (0.13 mol) of NMM are added to the stirred solution. The mixture is stirred overnight. Approx. 50 ml of benzine are added to the clear reaction solution and this mixture is then washed with 1 × 50 ml of deionized water, 3 × 50 ml of saturated $NaHCO_3$ solution, 1 × 50 ml of deionized water, 3 × 50 ml of 0.1 N HCl, 1 × 50 ml of deionized water and 12 × 20 ml of 3% $NaCO_3$ solution; it is then dried over 5 g of magnesium sulfate and evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature. 21.9 g of product, which solidifies in a glass-like manner, are obtained (yield: 100% of theory).

4. TFA*Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2

6 g (7.2 mmol) of Ddz-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2 are dissolved in 40 ml of 5% trifluoroacetic acid in DCM and the solution is stirred at RT for 1 h. After 40 ml

of toluene have been added, the mixture is evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature and subsequently codistilled with 40 ml of DME. The residue is dried under high vacuum. 3 g of foam are obtained (yield: quant.).

5. Ddz-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2

Liberation of Ddz-D-Trp from the DCHA salt:
6.56 g (11 mmol) of Ddz-D-Trp*DCHA are suspended in 20 ml of ethyl acetate and washed in a separating funnel with 3 × 20 ml of 0.1 N HCl and 2 × 10 ml of deionized water. The organic phase is dried over magnesium sulfate and evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature.

5.22 g (7.2 mmol) of TFA*Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2, the Ddz-D-Trp which was obtained above, and 1.57 g (10 mmol) of HOBT are dissolved in 40 ml of DME in a round-bottomed flask. 3.0 g (9.4 mmol) of TBTU and 3.4 ml (0.031 mol) of NMM are added to the stirred solution. The mixture is stirred overnight. The clear reaction solution is evaporated on a rotary evaporator at approx. 20 mBar, and 40°C water bath temperature and the residue is dissolved in 40 ml of ethyl acetate with approx. 10 ml of benzine then being added. The mixture is then washed with 1 × 20 ml of deionized water and 1 × 20 ml of 3% Na_2CO_3 solution. The product precipitates out and is filtered off with suction; it is then washed with PE/EE 2:1. After drying under high vacuum, 4.3 g of product are obtained (yield: 65% of theory).

6. TFA*D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2

11.2 g (11 mmol) of Ddz-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2 are dissolved in 64 ml of 5% trifluoroacetic acid in DCM and the solution is stirred at RT for 1 h. After

64 ml of toluene have been added, the mixture is evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature and subsequently codistilled with 60 ml of DME. After drying under high vacuum, the residue is triturated with ether. The precipitate is filtered off with suction and dried under high vacuum. 10 g of a beige solid are obtained (yield: 100%).

7. Ddz-Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2

Liberation of Ddz-Tyr(tBu) from the CHA salt:
7.38 g (13.2 mmol) of Ddz-D-Trp*DCHA are suspended in 30 ml of ethyl acetate and the suspension is washed in a separating funnel with 3 × 30 ml of 0.1 N HCl and 2 × 20 ml of deionized water. The organic phase is dried over magnesium sulfate and evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature.

10 g (11 mmol) of TFA*D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2, the Ddz-Tyr(tBu) which was obtained above, and 2.39 g (15.4 mmol) of HOBT are dissolved in 80 ml of N,N'-dimethylformamide in a round-bottomed flask. 4.59 g (14.3 mmol) of TBTU and 6 ml (0.055 mol) of NMM are added to the stirred solution. The mixture is stirred overnight. It is then evaporated on a rotary evaporator at approx. 40°C water bath temperature and the residue is taken up in 100 ml of ethyl acetate. This solution is then washed with 1 × 20 ml of deionized water, 3 × 30 ml of saturated $NaHCO_3$ solution, 1 × 20 ml of deionized water, 3 × 30 ml of cold 0.1 N HCl, 1 × 100 ml of brine and 3 × 20 ml of 3% Na_2CO_3 solution; it is then dried over approx. 5 g of magnesium sulfate and evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature. 11.8 g of amorphous product are obtained (yield: 87% of theory).

8. TFA*Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2

11.8 g (9.5 mmol) of Ddz-Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2 are dissolved in 50 ml of 5% trifluoroacetic acid in DCM and the solution is stirred at RT for 1 h. After 50 ml of toluene have been added, the mixture is evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature and subsequently codistilled with 50 ml of DME. After drying under high vacuum, the residue is triturated with ether. The precipitate is filtered off with suction and dried under high vacuum. 10.7 g of a beige solid are obtained (yield: 100%).

9. Ddz-Cys(Acm)-Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2

4.73 g (11 mmol) of Ddz-Cys(Acm), 10.7 g (50 mmol) of TFA*Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2 and 2.07 g (13 mmol) of HOBT are dissolved in 75 ml of N,N'-dimethylformamide in a round-bottomed flask. 3.97 g (12.4 mmol) of TBTU and 5.2 ml (0.048 mol) of NMM are added to the stirred solution. After having been stirred overnight, the mixture is evaporated on a rotary evaporator at approx. 40°C water bath temperature and the residue is taken up in ethyl acetate; the solution is washed with 1 × 30 ml of deionized water, 3 × 30 ml of saturated $NaHCO_3$ solution, 1 × 20 ml of deionized water, 3 × 20 ml of 0.1 N HCl, 1 × 20 ml of deionized water, 3 × 30 ml of saturated $NaHCO_3$ solution and 1 × 20 ml of deionized water; it is then dried over magnesium sulfate and evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature. 9.3 g of foam are obtained (yield: 70% of theory).

10. TFA*Cys(Acm)-Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2

8.9 g (6.3 mmol) of Ddz-Cys(Acm)-Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2 are dissolved in 35 ml of 5% trifluoroacetic acid in DCM and the solution is stirred at RT for 1 h. After 35 ml of toluene have been added, the mixture is evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature and subsequently codistilled with 60 ml of DME. After having been dried under high vacuum, the residue is triturated with 30 ml of ether and filtered off with suction; it is then washed and dried under high vacuum. 6.9 g of solid are obtained (yield: 84%).

11. Boc-D-Phe-Cys(Acm)-Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2

640 mg (2.4 mmol) of Boc-D-Phe, 2.61 g (2 mmol) of TFA*Cys(Acm)-Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2 and 430 g (2.8 mmol) of HOBT are dissolved in 10 ml of N,N'-dimethylformamide in a round-bottomed flask. 830 mg (2.6 mmol) of TBTU and 550 µl (10 mmol) of NMM are added to the stirred solution. After having been stirred overnight, the mixture is evaporated on a rotary evaporator at approx. 40°C water bath temperature; the residue is then taken up in ethyl acetate and the solution is washed with 1 × 10 ml of deionized water, 3 × 15 ml of saturated $NaHCO_3$ solution, 1 × 10 ml of deionized water, 3 × 10 ml of 0.1 N HCl, 1 × 10 ml of saturated $NaHCO_3$ solution and 1 × 10 ml of deionized water; it is then dried over magnesium sulfate and evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature. 2.1 g of amorphous residue are obtained (yield: 73% of theory).

12. TFA*D-Phe-Cys(Acm)-Tyr-D-Trp-Lys-Cys(Acm)-Thr-NH_2

1.44 g (1 mmol) of Boc-D-Phe-Cys(Acm)-Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2 are dissolved in 7.5 ml of trifluoroacetic acid and the solution is stirred at RT for 16 h. After checking by TLC, precipitation is carried out in 75 ml of diethyl ether. The precipitate is filtered off with suction, washed and dried under high vacuum. 1.26 g of powder are obtained (yield: 88%).

13. TT-232 trifluoroacetate

300 ml of acetic acid (96%) are initially introduced into a 1 l round bottomed flask; 0.215 g (0.15 mmol) of TFA*D-Phe-Cys(Acm)-Tyr-D-Trp-Lys-Cys(Acm)-Thr-NH_2 is then added slowly while stirring thoroughly. The solution is degassed with argon. A solution of 92 mg (0.36 mmol) of iodine in 6 ml of MeOH is added slowly dropwise, at 22°C and through a dropping funnel, until the reaction solution assumes a stable, slightly orange color. After 1 h, a solution of 53 mg (0.3 mmol) of ascorbic acid in 5 ml of deionized water is added until the reaction solution has lost its color. The solution is evaporated on a rotary evaporator at 40°C water bath temperature; after having been dried under high vacuum, the residue is then triturated with 5 ml of ether, filtered off with suction, washed and dried under high vacuum. 280 mg of solid are obtained (HPLC comparison with a reference indicates a content of 53%).

Example 5

The procedure is as described in Example 4 except that the disulfide bridge is linked while the peptide is protected.

1. Tert-butyl-protected TT232

72 mg (0.05 mmol) of Boc-D-Phe-Cys(Acm)-Tyr(tBu)-D-Trp-Lys(Z)-Cys(Acm)-Thr(tBu)-NH_2 are added slowly, while stirring thoroughly, to 100 ml of acetic acid (96%) in a round bottomed flask. The solution is degassed with argon. A solution of 92 mg (0.36 mmol) of iodine in 6 ml of MeOH is slowly added dropwise, at 22°C and through a dropping funnel, until the reaction solution assumes a stable, slightly orange color. After 1 h, a solution of 53 mg (0.3 mmol) of ascorbic acid in 5 ml of deionized water is added until the reaction solution has lost its color. The solution is then evaporated on a rotary evaporator at 40°C water bath temperature and the residue is taken up in 5 ml of ethyl acetate and 2 ml of deionized water; the organic phase is washed with 3 × 2 ml of saturated $NaHCO_3$ solution, 1 × 2 ml of deionized water, 1 × 2 ml of 0.1 N HCl and 1 × 10 ml of deionized water; it is then dried over magnesium sulfate and evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature. After drying under high vacuum, 45 mg of residue are obtained (yield: 71% of theory).

2. TT232 trifluoroacetate

39 mg (0.03 mmol) of tert-butyl-protected TT232 are dissolved in 230 µl of trifluoroacetic acid and the solution is stirred at RT for 16 h. It is then evaporated on a rotary evaporator at approx. 20 mbar and 40°C water bath temperature. After having been dried under high vacuum, the residue is triturated with 1 ml of ether and filtered off with suction; it is then washed and dried under high vacuum. 33 mg of product are obtained (yield: 77%).

Patent claims

1. A process for preparing Biostatin (TT 232) by means of peptide synthesis in solution, by synthesizing the peptide stepwise using protective group-derivatized amino acids,
characterized in that
the disulfide bridge is closed by means of oxidizing the completely or partially synthesized peptide with iodine in the presence of a suitable solvent and the Biostatin is obtained after removing the solvent and, where appropriate, washing.

2. The process as claimed in claim 1,
characterized in that
the oxidation is carried out before all the protective groups are eliminated.

3. The process as claimed in claim 1 or 2,
characterized in that
Ddz residues (3,5-dimethoxybenyl-α,α-dimethyloxy-carbonyl or [2(3,5-dimethoxyphenyl)-2-oxy-carbonyl]propyl) are used as protective groups for one or more of the amino acids.

4. A process for preparing Biostatin (TT 232) by means of peptide synthesis in solution, by synthesizing the peptide stepwise using protective group-derivatized amino acids,
characterized in that
the disulfide bridge is closed by oxidizing the completely or partially synthesized peptide in the presence of a suitable solvent before eliminating all the protective groups and the Biostatin is obtained after removing the solvent and, where appropriate, washing.

5. The process as claimed in claim 4,
characterized in that
Ddz residues are used as protective groups for one or more of the amino acids.

6. A process for preparing Biostatin (TT 232) by means of peptide synthesis in solution, by synthesizing the peptide stepwise using protective group-derivatized amino acids,
characterized in that
the disulfide bridge is closed by oxidizing the completely or partially synthesized peptide in the presence of a suitable solvent and the Biostatin is obtained after removing the solvent and, where appropriate, washing, with Ddz residues being used as protective groups for one or more of the amino acids.

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My resident, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled " Process for preparing BIOSTATIN (TT-232 triacetate) and its analogs ", the specification of which

( ) is attached hereto.
(x) was filed on 20 Aug. 1999 as PCT International Application Serial No. PCT/EP99/06131

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

Foreign Priority Applications

I hereby claim foreign priority benefits under Title 35, United States Code 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

			Priority Claimed
PCT/EP98/05306 (Number)	WO (Country)	20.08.1998 (Day/Month/Year Filed)	Yes (x) No ( )
(Number)	(Country)	(Day/Month/Year Filed)	Yes ( ) No ( )

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)

U.S. Priority Applications

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Applic. Serial No.)	(Filing Date)	(Status-patented/pending/abandoned)
(Applic. Serial No.)	(Filing Date)	(Status-patented/pending/abandoned)

Power of Attorney

I hereby appoint the following attorneys and patent agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Peter F. Felfe, Reg. No. 20,297; John E. Lynch, Reg. No. 20,940; Norman D. Hanson, Reg. No. 30,946; John A. Bauer, Reg. No. 32,554; Mary Anne Schofield, Reg. No. 36,669; James Zubok, Reg. No. 38,671; James R. Crawford, Reg. No. 39,155, Andrew Im, Reg. No. 40,657 and David Rubin, Reg. No. 40,314; my attorneys with full power of substitution and revocation.

Customer No. 24972

Address all telephone calls to _____ at (212) 318-3000.

Address all correspondence to: FULBRIGHT & JAWORSKI L.L.P.
666 Fifth Avenue
New York, New York 10103

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(1) BRAUM, Günther — Signature — 8.1.2001
Full Name/Sole or First Inventor — Signature — Date

Residence: Jakobsgasse 1
69214 Eppelheim, Germany DEX

Post Office Address: same as above

Citizenship: German

(2) LIEFERTH, Axel — Signature — 8.01.2001
Full Name/Second Inventor — Signature — Date

Residence: Keplerstraße 23
69207 Sandhausen, Germany DEX

Post Office Address: same as above

Citizenship: German

(3) over

x [signature] 8. Jan. 01

3-00 (3) BIRR, Christian
Full Name/Third Inventor Signature Date

Residence: Bahnhofstraße 130
69151 Neckargmünd DEX

Post Office Address: same as above

Citizenship: German

(4)
Full Name/Fourth Inventor Signature Date

Residence:

Post Office Address:

Citizenship:

(5)
Full Name/Fifth Inventor Signature Date

Residence:

Post Office Address:

Citizenship: